BRADYKININ B₂ RECEPTOR ACTIVATES EXTRA-CELLULAR SIGNAL
REGULATED PROTEIN KINASE IN mIMCD-3 CELLS via EPIDERMAL
GROWTH FACTOR RECEPTOR TRANSACTIVATION.

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- d) The abbreviations used are: **BK**, bradykinin; **CaM**, calmodulin; **DMEM**, Dulbecco's modified Eagle's medium; **EGF**, epidermal growth factor; **EGFR**, epidermal growth factor receptor; **ERK**, extracellular signal regulated protein kinase; **F12**, Ham's F12 nutrient mixture; **GPCR**, G protein-coupled receptors; **Grb2**, growth factor receptor binding protein 2; **Jak2**, Janus kinase 2; **LPA**, lysophosphatidic acid; **MAPK**, mitogen-activated protein kinase; **MEK**, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; **mIMCD-3**, inner medullary collecting duct cells; **PBS**, phosphate-buffered saline; **PI3K**, phosphatidylinositol 3-kinase; **PKC**, protein kinase C; **PLC**, phospholipase

C; **PMA**, phorbol-12-myristate-13-acetate; **PTX**, pertussis toxin; **RIPA**, radioimmune precipitation buffer; **RTK**, receptor tyrosine kinase; **Shc**, Src homology and collagen protein; **Sos**, son of sevenless (Ras exchange factor); **VSMC**, vascular smooth muscle cells.

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ABSTRACT

Bradykinin (BK) has been implicated in the regulation of renal function. Activation of extracellular signal-regulated protein kinase (ERK1/2) has been demonstrated in several models of toxic or proliferative renal injury. We studied activation of ERK1/2 by BK in a cell model of the most distal part of nephron, inner medullary collecting duct (mIMCD-3) cells. Exposure of mIMCD-3 cells to BK (10^{-10} - 10^{-5} M) resulted in concentration-dependent increase in tyrosine phosphorylation of ERK1/2 with maximal effect at 10^{-8} M of BK. ERK1/2 activation by BK was observed as early as 1 min, peaked at 5 min, and was sustained at least for 1 hour. The effect of BK was mediated by the B₂ receptor and was pertussis toxin-independent. Inhibition of phospholipase C, protein kinase C or phosphatidylinositol 3-kinase did not alter ERK1/2 activation by BK. BK-induced ERK1/2 activation was Ca²⁺-calmodulin independent but was sensitive to genistein, an inhibitor of tyrosine kinase(s). AG1478, a specific inhibitor of epidermal growth factor receptor (EGFR) kinase, completely blocked the effect of BK suggesting an essential role of EGFR in ERK1/2 activation by BK. Immunoprecipitation/ Western blot studies revealed that BK stimulated tyrosine phosphorylation of EGFR, its association with an adapter molecule Grb2, and complex formation between Grb2 and the adapter protein Shc. Activation studies of monomeric G protein Ras showed that BK-induced stimulation of Ras was dependent on EGFR tyrosine kinase activity.

These studies demonstrate that BK stimulates Ras-dependent activation of ERK1/2 in mIMCD-3 cells via transactivation of EGFR through a novel mechanism.

The vasoactive nonapeptide bradykinin (BK) has been implicated in the regulation of kidney function, particularly in electrolyte and water excretion (Vio et al., 1992; Mukai et al., 1996), but the cellular mechanisms by which BK alters functions of renal cells are not completely understood. In the inner medullary collecting duct (IMCD), BK regulates sodium excretion and, consequently, controls extracellular fluid volume (Tomita et al., 1985). We recently showed that BK stimulates Na⁺/H⁺ exchanger type 1, which is essential for regulation of cell growth, intracellular pH, and cellular volume, in a cultured murine cell model of IMCD (mIMCD-3 cells) (Mukhin et al., 2001). Because BK has been implicated in the regulation of mitogen-activated protein kinase (MAPK) in various cells (Liebmann, 2001), we proposed to study its possible role in regulation of extracellular signal-regulated protein kinase (ERK1/2) in kidney cells. ERK1/2 belongs to the MAPK family of serine/threonine kinases that are rapidly activated in response to growth factor stimulation (Cobb & Goldsmith, 1995). ERK1/2 may be regulated by both receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs). ERK1/2, after being activated through phosphorylation of threonine²⁰² and tyrosine²⁰⁴ by MEK1/2, translocates to the nucleus and activates transcription factors, thereby regulating cell proliferation (Widmann, et al., 1999). The mechanism of ERK stimulation by GPCRs appears to be complex. Receptors coupled to G_i proteins usually cause ERK activation via a G_{βγ} subunit - mediated pathway, which involves activation of Ras and consecutive stimulation of Raf and MEK1/2. Other GPCRs mediate ERK activation via a G_q subunit

pathway that is Ras-independent and involves protein kinase C (PKC) (Gutkind, 1998). In some systems Ca^{2+} /calmodulin (CaM) has also been implicated in G_i - and G_q -coupled receptor-mediated ERK activation (Della Rocca et al., 1997). Another pathway used by GPCRs to activate ERK involves “transactivation” of growth factor receptors with intrinsic tyrosine kinase activity, such as the epidermal growth factor receptor (EGFR) (Daub et al., 1997; Carpenter, 1999). In this case, stimulation of G_i - or G_q -coupled receptors leads to activation of the EGFR tyrosine kinase, which in turn leads to the activation of Ras and initiates the phosphorylation cascade involving Raf, MEK1/2, and ERK.

The bradykinin B_2 receptor, a prototypical GPCR, (McEachern et al., 1991) has been shown to activate ERK1/2 in vascular smooth muscle cells (VSMC) (Velarde et al., 1999), endothelial cells (Bernier et al., 2000), PC-12 cells (Dikic et al., 1996), and various tumor cell lines (Drube & Liebmann, 2000; Graness et al., 2000). There is contradictory evidence in the literature about the mechanisms through which BK induces ERK1/2 activation. In VSMC, BK stimulates ERK1/2 via the B_2 receptor, PKC, and the non-receptor tyrosine kinase Src (Velarde et al., 1999). A role for Ca^{2+} /CaM in BK-induced activation of ERK1/2 in VSMC has also been proposed (Naidu et al., 1999). In PC-12 cells BK also uses a Ca^{2+} -dependent pathway that involves the non-receptor tyrosine kinases Pyk2 and Src to activate ERK1/2 (Dikic et al., 1996). In carcinoma cell lines SW-480 and A431, BK-induced ERK1/2 activation involves phosphatidylinositol 3-kinase (PI3K) and PKC (Graness et al., 1998; 2000). In other tumor cell lines BK activates mitogenic pathways via pertussis toxin (PTX)-sensitive $\text{G}_{i/o}$ proteins, PI3K and

PKC (Drube & Liebmann, 2000). Finally, the B₂ receptor transfected into COS-7 cells uses two different pathways to activate ERK1/2: a PKC-dependent pathway and EGFR transactivation (Adomeit et al., 1999).

Little is presently known about the role of BK in ERK1/2 signaling in kidney cells. However, it is extremely important because studies employing in vivo renal model systems demonstrated connection of inflammatory or toxic renal injury with the activation of ERK1/2 in renal tissue (Tian et al., 2000). Activation of ERK1/2 by BK in kidney cells has been demonstrated for cultured mesangial cells, although its mechanism remains unclear (El-Dahr et al., 1998; Jaffa et al., 1997). Even less is known about ERK1/2 regulation by BK in renal tubular epithelial cells. The only paper published on this topic shows that BK activates ERK1/2 in rabbit cortical collecting duct cells via a PKC-dependent mechanism (Lal et al., 1998).

Here we used mIMCD-3 cells, a cell culture model of the terminal segment of the nephron, where the final urinary composition is made. mIMCD-3 cells are able to survive in high concentrations of NaCl and urea, and urea-inducible ERK1/2 activation has been shown in these cells (Yang et al., 1999). In the present study we demonstrate for the first time that BK activates ERK1/2 in mIMCD-3 cells and describe the signal transduction pathway of this activation.

METHODS

Cell Culture

The mIMCD-3 cells (Rauchman et al., 1993) were obtained from American Type Culture Collection (Rockville, MD). mIMCD-3 cells were grown in an equal part mixture of DMEM and Ham's F12 supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin in a cell culture incubator at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

ERK Assays

ERK activity was assessed using a phosphorylation state specific ERK antibody (Cell Signaling Technology Inc., Beverly, MA, USA) which specifically recognizes threonine²⁰²- and tyrosine²⁰⁴-phosphorylated (but not non-phosphorylated) ERK-1 and ERK-2 and does not react with other members of the MAPK family, such as ERK-5, p38 MAPK or stress-activated protein kinases.

Cells were grown in 12- well plastic culture plates to 80% confluence and serum starved for 48 hours in DMEM/F12 medium with 0.5% BSA, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Treatments with different inhibitors were carried out for 30 minutes prior to stimulation with BK for 5 minutes. After treatments, cells were scraped into Laemmli buffer and subjected to SDS-PAGE under reducing conditions using 4-20% pre-cast gels (Novex, San Diego, CA, USA). After semi-dry transfer to polyvinylidene difluoride membranes, the membranes were blocked with a BLOTTO buffer and incubated with the phospho-ERK antibody (at 1:1000 dilution). After incubation with goat anti-rabbit

alkaline phosphatase-conjugated IgG, immunoreactive bands were visualized by a chemiluminescent method (CDP StarTM, New England Biolabs) using Kodak X-AR film, and quantified using a GS-670 densitometer and Molecular Analyst software (BioRad).

The same membranes were stripped using Re-Blot Plus Western blot recycling kit (Chemicon International, Temecula, CA) and re-probed with the control ERK antibody, which recognizes equally well the phosphorylated and non-phosphorylated ERK to quantify total ERK1/2. Results were presented as intensities of phospho-ERK1/2 bands relative to total ERK1/2 and expressed as percent of control (cells without BK treatment) phosphorylation.

Study of EGFR phosphorylation and association with B₂ receptor and Grb2

The phosphorylation state of EGFR and its association with the adapter protein Grb2 were assessed by immunoprecipitation / Western blotting studies. Quiescent cells, grown in 100 mm dishes, were treated with 10 nM of BK or with 10 ng/ml or 1 ng/ml EGF for 5 minutes and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na₃V0₄, 1 µg/ml each of aprotinin, leupeptin, and pepstatin). Cell lysates were pre-cleared by incubating with protein A-agarose bead slurry for 30 min at 4°. Pre-cleared lysates (1µg/µl total cell protein) were incubated with 4 µg of anti-EGFR polyclonal IgG (UBI, Lake Placid, NY) overnight at 4°. The immunocomplexes were captured by addition of protein A-agarose bead slurry and incubation for 2 more hours at 4°. The agarose beads were collected by

centrifugation, washed 3 times with RIPA buffer, resuspended in 2X Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE. After semi-dry transfer to polyvinylidene difluoride membranes, the membranes were probed with monoclonal anti-phospho-EGF receptor antibodies (UBI, Lake Placid, NY) to assess the phosphorylation state of EGFR or with monoclonal anti-Grb2 antibodies (UBI, Lake Placid, NY) to study the presence of Grb2 in EGFR immunoprecipitates. To examine the possibility that the B₂ receptor associates with EGFR in order to activate it, we also performed Western blotting with monoclonal anti- B₂ receptor antibody (Research Diagnostics Inc, Flanders, NJ).

Association of Grb2 with Shc

Association of Grb2 with the adapter protein Shc was assessed by immunoprecipitation of cell lysates from mIMCD-3 cells treated with vehicle, 10 nM of BK, or 1 ng/ml EGF for 5 minutes with polyclonal anti-Shc antibody (UBI, Lake Placid, NY) followed by Western blotting with monoclonal anti-Grb2 antibodies (UBI, Lake Placid, NY).

Ras Activation Assay

Ras activation was assessed by a non-radioactive Ras assay kit (UBI, Lake Placid, NY). Quiescent mIMCD-3 cells monolayers were pretreated with AG1478 or vehicle for 30 minutes, stimulated with 10 nM BK, 1 ng/ml EGF or vehicle for 5 minutes, lysed in 1 ml/100 mm dish of Mg²⁺ Lysis buffer (MLB) (150 mM NaCl, 25 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM MgCl₂, 1% Igepal CA-

630, 25 mM NaF, 1 mM Na₃VO₄, aprotinin and leupeptin at 10 µg/ml of each, 10 % glycerol). Cell lysates were pre-cleared by incubating with glutathione agarose for 10 min at 4°C. Pre-cleared lysates (1 µg/µl total cell protein) were incubated with 10 µg of Raf-1 RBD (GST fusion-protein, corresponding to Ras Binding Domain of Raf-1) and bound to glutathione agarose for 30 minutes at 4°C. The agarose beads were collected by centrifugation, washed 3 times with MLB buffer, resuspended in 2× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE and subsequent immunoblot analysis with monoclonal anti-Ras IgG.

DNA Synthesis

DNA synthesis was assessed by measuring the incorporation of [³H]thymidine into DNA fragments. Quiscent mIMCD-3 cells grown in 24-well plates were stimulated for 18 hours with 100 nM BK or with 10 ng/ml EGF in the presence and absence of MEK1 inhibitor PD98059 (50 µM) or EGFR tyrosine kinase inhibitor AG-1478 (100 nM). The cells were labeled for 6 hours with 1µCi/ml per well of [³H]thymidine. Then, after 2 saline washes, 0.5 ml of 0.3 M perchloric acid was added for 30 seconds, followed by saline wash and solubilization in 0.1%SDS/0.1N NaOH. Cells were scraped into vials, and incorporated radioactivity was quantified in a scintillation counter.

Data Analysis

ERK assays were performed in duplicate and repeated at least 3 times. EGFR assays and Ras activation assays were repeated 3 times for each

condition. Thymidine incorporation assays were performed in triplicates and repeated at least 4 times. Data are presented as mean $\pm$ S.E.M. (Standard Error of the Mean) and were analyzed for repeated measures by Student's *t*-test for unpaired 2-tailed analysis. Differences were considered significant at $P < 0.05$. Plots and graphs were prepared with SigmaPlot 4.0 for Windows (SPSS Inc., San Rafael, CA). Nonlinear regression analysis was done using SigmaPlot 4.0 Regression Wizard with user-modified functions.

RESULTS

Activation of ERK by BK. Treatment of mIMCD-3 cells with 10 nM of BK resulted in a time-dependent increase in tyrosine phosphorylation of ERK1/2 (Figure 1). BK treatment produced a 4- to 5-fold increase in ERK phosphorylation over basal activity. ERK phosphorylation was detectable as early as 1 minute, peaked at 3-5 minutes, and was sustained at least for 1 hour. BK stimulated ERK activation in a concentration-dependent manner, with maximal activation at 10^{-8} M (Figure 2). In subsequent experiments ERK assays usually were carried out with stimulation by 10 nM BK for 5 minutes, unless otherwise mentioned.

BK stimulates ERK through a B_2 receptor. mIMCD-3 cells were pretreated for 30 minutes with a B_1 receptor antagonist des-Arg¹⁰-HOE-140 (1 μ M) or with a B_2 receptor antagonist HOE-140 (1 μ M), then stimulated with 100 nM of BK for 5 minutes. Figure 3 shows that pretreatment with HOE-140 prevented the BK-induced activation of ERK, whereas a B_1 receptor antagonist was without effect. Thus, BK stimulates ERK activity via the B_2 receptor.

BK receptors do not couple to $G_{i/o}$ proteins in mIMCD-3 cells. mIMCD-3 cells were preincubated with vehicle or pertussis toxin (PTX, 200ng/ml) overnight, then stimulated with 10 nM BK or 1 μ M lysophosphatidic acid (LPA) for 5 minutes. Figure 4 shows that there was no difference in BK-induced ERK phosphorylation in PTX-treated and non-treated cells, whereas LPA-induced signal was inhibited by ~ 45%. LPA has been previously shown to use PTX-sensitive intracellular signaling pathways (Seewald et al., 1999; Goppelt-Struebe

et al., 2000), so this result supports that PTX-sensitive $G_{i/o}$ α subunits were indeed inhibited under our experimental conditions.

Effect of MEK inhibitors on BK stimulation of ERK. Next, the effect of 2 different inhibitors of ERK kinase (MEK) on BK stimulation of ERK tyrosine phosphorylation was investigated. Pretreatment of mIMCD-3 cells with 10 μ M PD 098059 and with 10 μ M U0126 for 30 minutes prior to stimulation with 10 nM of BK for 5 minutes abolished BK-stimulated tyrosine phosphorylation of ERK1/2 (Figure 5). These results confirm that in mIMCD-3 cells BK stimulation of ERK proceeds, as expected, through MEK.

Lack of involvement of PLC and PKC in BK-induced activation of ERK. Different chemical inhibitors of PLC and protein PKC were used to examine the role of these proteins in BK-induced activation of ERK in mIMCD-3 cells. The PKC inhibitor GF109203X (1 μ M for 30 minutes) as well as PKC depletion by the prolonged (20 hours) treatment of the cells with 160 nM phorbol 12-myristate 13-acetate (PMA) did not block BK-stimulated ERK activation. At the same time both treatments inhibited ERK activation by PMA, showing that PKC was indeed inhibited under our experimental conditions (Figure 6A). That result is consistent with the non-involvement of PKC in BK-stimulated activation of ERK. Cells were also pretreated with vehicle, or with 3 different PLC inhibitors [U-73122 (10 μ M), D609 (50 μ M), and ET-18-OCH₃ (50 μ M)] for 30 minutes, and then stimulated with 10 nM BK for 5 minutes. Treatment with PLC inhibitors slightly elevated the basal ERK activity but did not impair the BK-stimulated activation of ERK, suggesting that PLC is not involved in this process (Figure 6B).

Lack of involvement of Calcium and Calmodulin in BK-induced ERK activation. To examine the role of calcium in BK-induced ERK activation, cells were pretreated for 30 minutes with 10 μ M of BAPTA, a cell-permeable Ca^{2+} sequestrant. To determine whether CaM activity is required for BK-mediated ERK activation, cells were pretreated for 30 minutes with CaM antagonist, fluphenazine (50 μ M). As in the case of PLC inhibitors, both treatments caused a slight increase in a basal ERK activity. At the same time, neither of these treatments impaired BK-stimulated ERK activation, indicating that Ca^{2+} and CaM are not important for BK stimulation of ERK in mIMCD-3 cells (Figure 7).

Role of PI3 kinase and tyrosine kinases. Because activation of ERK typically requires activation of phosphorylation cascades, the role of PI3K as well as different tyrosine kinases in the effect of BK was examined. Two different specific inhibitors of PI3K (50 nM wortmannin and 50 μ M LY294002) were used to pretreat mIMCD-3 cells for 30 minutes before stimulation with 10 nM BK for 5 minutes. Both pre-treatments did not alter the ability of BK to stimulate ERK activity (Figure 8), suggesting the non-involvement of PI3K in BK-induced ERK activation in mIMCD-3 cells.

Genistein (50 μ M), a broad-spectrum tyrosine kinase inhibitor, markedly attenuated the ability of BK to activate ERK, whereas the structurally similar but inactive compound, daidzein, had no effect (Figure 8). Another inactive analog of genistein, genistin (50 μ M) was also without effect (data not shown). These results suggest the involvement of a tyrosine kinase in the pathway of ERK activation by BK. Next, several tyrosine kinase inhibitors selective for different

tyrosine kinases were used in order to find out which of them is involved in the BK-induced activation of ERK. The specific inhibitor of non-receptor Janus kinase 2 (AG-490, 50 μ M) had no effect on the BK-induced activation of ERK. At the same time AG1478 (100 nM), a selective inhibitor of EGFR tyrosine kinase effectively blocked the BK-induced activation of ERK, suggesting that activation of EGFR is involved in the BK-induced ERK activation in mIMCD-3 cells.

BK induces phosphorylation of EGF receptor. Because GPCR-induced tyrosine phosphorylation (transactivation) of EGFR has been recently described (Daub et al., 1997; Carpenter, 1999), we next studied the possibility that the B₂ receptor induces phosphorylation of the EGFR in mIMCD-3 cells. We performed immunoprecipitation/Western blotting studies to assess the phosphorylation state of EGFR and demonstrated that BK induced a time-dependent ~2 fold increase in EGFR phosphorylation that started as early as 1 minute after BK application. Pre-treatment of mIMCD-3 cells with 100 nM of AG1478 (selective inhibitor of EGFR tyrosine kinase) completely blocked BK-induced phosphorylation of EGFR (Figure 9).

BK does not stimulate complex formation of B₂ receptor with EGFR. One of the mechanisms of GPCR-induced transactivation of EGFR that has been recently described for the β_2 -adrenergic receptor involves the complex formed between EGFR and the β_2 -adrenergic receptor (Maudsley et al., 2000). We considered the possibility that the same mechanism exists in mIMCD-3 cells and employed immunoprecipitation/Western blotting studies to test it. However, there

was no B₂ receptor in EGFR-immunoprecipitates from mIMCD-3 cells either non-stimulated or stimulated with 10 nM BK (Figure 10).

BK stimulates complex formation of Grb2 with EGFR and association of Grb2 with Shc in mIMCD-3 cells. Tyrosine phosphorylation of EGFR usually leads to activation of ERK1/2 via consecutive recruitment or activation of the adapter proteins Grb2 and/or Shc, the small GTP-binding protein Ras, and a cascade of protein kinases consisting of Raf, MEK, and ERK. Figure 10 demonstrates that treatment of mIMCD-3 cells with 10 nM BK for 5 minutes stimulates complex formation of EGFR with Grb2 (Figure 11A) and also increased the amount of the adapter protein Shc associated with Grb2 (Figure 11B).

BK activates Ras in mIMCD-3 cells in EGFR-dependent manner. Using a direct measurement of Ras activation in mIMCD-3 cells treated with BK, we found that 10 nM of BK caused time-dependent activation of Ras. This activation occurs as early as 1 minute after BK application and peaks at 2.5 minutes (Figure 12A). Pretreatment of the cells with 100 nM AG1478, which blocks EGFR tyrosine kinase, completely prevented Ras activation by BK and/or EGF (Figure 12B), supporting an important role for EGFR tyrosine kinase activity in this process.

BK-induced DNA synthesis in mIMCD-3 cells depends on EGFR and activation of ERK. The mitogenic effect of BK in mIMCD-3 cells was evaluated by measuring DNA synthesis. There was a significant increase in [³H]thymidine incorporation in BK-treated cells (87 ± 19% over control values; P < 0.01, n=6)

which was comparable to EGF-induced DNA synthesis ($102 \pm 32\%$ over control; $P < 0.05$, $n=6$). To assess a role for EGFR tyrosine kinase and ERK in the mitogenic actions of BK, cells were pretreated with 100 nM AG1478 (EGFR tyrosine kinase inhibitor) or with 50 μ M PD98059 (MEK1 inhibitor) for 1 hour before stimulation with 100 nM BK or 10ng/ml EGF for 18 hours in the continuous presence of the inhibitors. Figure 13 shows that each inhibitor completely blocked the increase in [3 H]thymidine incorporation induced by BK or EGF. AG1478 and PD98059 alone had no significant effect on the basal rate of DNA synthesis. These findings directly demonstrate that BK stimulates proliferation of mIMCD-3 cells and further implicate a role for activation of EGFR tyrosine kinase and ERK1/2 in the BK mitogenic activity.

DISCUSSION

In spite of an obviously important role of BK in the kidney, the processes by which BK alters functions of renal cells are not completely understood. Because BK acts as a mitogenic factor in different cell types (Liebmann, 2001) and because aberrant ERK1/2 regulation is implicated in several models of proliferative or toxic renal injury (Tian et al., 2000), we studied a role of BK in the regulation of ERK1/2 in kidney cells. Using a cultured murine cell model of the IMCD (mIMCD-3 cells), we showed that BK induces time- and concentration-dependent tyrosine phosphorylation of ERK1/2 (Figure 1,2).

Next, the signal transduction pathway that leads to ERK1/2 activation by BK was studied. BK-induced ERK1/2 activation was blocked by a B₂ bradykinin receptor antagonist indicating that BK activates ERK1/2 via a B₂ receptor, a member of the seven transmembrane GPCR superfamily (McEachern et al., 1991). Our data suggest that PTX-sensitive GTP-binding proteins (G_{i/o}) are not involved in BK-induced ERK1/2 activation, which is different from the pathway described in human carcinoma cell lines, where BK employs G_{i/o} proteins to activate ERK1/2 (Drube & Liebmann, 2000). B₂ receptor usually couples to the GTP-binding protein G_q, and stimulates PLC activity, leading to the generation of inositol 1,4,5-triphosphate and diacylglycerol, later of which is involved in activation of PKC (Bascands et al., 1991). Activation of PLC also leads to an increase in intracellular calcium, which in turn can stimulate different signaling targets inside the cell (Dixon et al., 1994). It has been previously published that BK stimulates ERK1/2 activation in VSMC via PKC-dependent and Ca²⁺/CaM

dependent mechanism (Velarde et al., 1999; Naidu et al., 1999). A PKC-dependent pathway of BK-induced ERK1/2 activation has been also described in transfected COS cells and multiple tumor cell lines (Liebmann, 2001). It seemed reasonable to expect that BK would use the similar pathway to activate ERK1/2 in mIMCD-3 cells. It appears however, that inhibition of PLC or PKC did not alter ERK1/2 activation by BK, suggesting the non-involvement of these proteins in the signal transduction pathway (Figure 6). We also demonstrated that Ca^{2+} and CaM are not important for BK stimulation of ERK1/2 in mIMCD-3 cells (Figure 7). Thus, in mIMCD-3 cells, in contrast to other cell lines, BK activates ERK1/2 *via* PKC- and Ca^{2+} /CaM-independent pathway, indicating that in different types of cells BK can induce different signaling pathways that result in ERK1/2 activation.

Because the signaling pathways that link the B_2 receptor to ERK1/2 in other cells also were shown to involve PI3K (Graness et al., 1998), we thought that the B_2 receptor might stimulate ERK1/2 via activation of PI3K. However, two different inhibitors of PI3K had no effect on the BK-induced activation of ERK1/2, suggesting a lack of PI3K involvement in the signaling pathway (Figure 8). Next, we assumed that the B_2 receptor would use tyrosine kinase(s)-dependent pathway to stimulate ERK. Genistein, an isoflavenoid which is commonly used as a broad specificity tyrosine kinase inhibitor, significantly suppressed the increase in ERK1/2 phosphorylation in response to BK stimulation. Because genistein has been reported to have a wide range of biological activities, we also used its inactive analogs daidzein and genistin, neither of which had any effect on BK-

induced ERK1/2 activation. Furthermore, the effects of more specific cell-permeable inhibitors of non-receptor Janus kinase (Jak2), and EGFR tyrosine kinase were examined. Although it has been shown recently that BK activates Jak2 in mIMCD-3 cells (Mukhin et al., 2001), our data did not support the involvement of Jak2 in BK-induced ERK signaling (Figure 8). On the other hand, the EGFR kinase inhibitor AG1478 eliminated the increase in ERK1/2 phosphorylation induced by BK. These findings support a role of EGFR kinase in the signal transduction pathway leading to ERK1/2 activation by BK. This phenomenon has been recently described for many GPCRs (Daub et al., 1997; Carpenter, 1999). EGFR is a growth factor receptor with intrinsic tyrosine kinase activity. EGF binds to its receptor at the plasma membrane and activates it through a mechanism that involves dimerization, activation of the receptor tyrosine kinase, and autophosphorylation of the receptor. Activated EGFR then stimulates ERK1/2 through a chain of events that includes binding to the adapter protein Grb2, tyrosine phosphorylation of the adaptor protein Shc, and subsequent formation of Shc-Grb2-Sos1 complex. The Ras guanine nucleotide exchange factor Sos1 stimulates small G protein Ras, which in turn initiates the phosphorylation cascade consisting of protein kinases Raf, MEK, and ERK.

We employed immunoprecipitation/Western blot studies and showed that in mIMCD-3 cells BK indeed stimulated time-dependent tyrosine phosphorylation of EGFR that was dependent on the kinase activity of EGFR (Figure 9), induced complex formation between EGFR and Grb2, and stimulated association of Grb2 with the adaptor protein Shc (Figure 11A,B). Finally, we showed that stimulation

of mIMCD-3 cells with BK caused time-dependent activation of Ras, that occurred as early as 1 minute after BK application, and peaked at 2.5 minutes (Figure 12A), slightly preceding the peak activation of ERK, consistent with a potential role for Ras upstream of ERK. This BK-stimulated Ras activation was comparable with Ras activation induced by EGF and was also dependent on EGFR tyrosine kinase activity (Figure 12B). Thus, our results provide evidence that the B₂ receptor activates ERK1/2 via a pathway that shares mediators of growth signaling initiated by EGF, including activation of EGFR, Grb2, Shc, Ras, and MEK1/2, similar to a pathway described for other GPCRs (Carpenter, 1999).

The mechanisms underlying GPCR-induced EGFR transactivation have not been clearly defined. One transactivation pathway recently proposed for the β_2 -adrenergic (β_2 -AR) receptor signaling includes the isoproterenol-induced formation of a β_2 -AR-EGFR complex (Maudsley et al., 2000). We considered the possibility that BK stimulation induces a complex formation between the B₂ receptor and EGFR in mIMCD-3 cells. However, our experimental data do not support this possibility (Figure 10B).

Several mechanisms of EGFR transactivation by G_q-coupled receptors have been demonstrated. A metalloprotease-dependent EGFR activation by heparin-binding EGF-like growth factor was described for the angiotensin II AT₁ receptor in vascular smooth muscle cells (Eguchi et al., 2001) and glomerular mesangial cells (Uchiyama-Tanaka et al., 2001) and for the muscarinic M₁ receptor and thrombin receptor (Prenzel et al., 1999; Kalmes et al., 2000). Another published mechanism of EGFR transactivation that involves Ca⁺²-

dependent tyrosine kinase PYK-2 and Src kinase has been demonstrated for AT₁ receptor in VSMC (Eguchi et al., 1999) and for the muscarinic M₃ receptor in T₈₄ intestinal epithelial cells (Keely et al., 2000). A third, PKC-dependent mechanism of EGFR transactivation has been shown for the receptor of gonadotropin releasing hormone in pituitary α T3-1 gonadotropes and in transfected COS-7 cells (Grosse et al., 2000) and for M₁ muscarinic acetylcholine receptors transfected into HEK-293 cells (Tsai et al., 1997). Finally, the last mechanism of G_q-coupled receptors-dependent EGFR transactivation described for the thromboxane A₂ (TxA₂) receptor involves stimulation of PKC through receptor - G_q coupling and PLC activation. Activated PKC then phosphorylates the TxA₂ receptor directly or a mediator, facilitating TxA₂ receptor coupling to G_i, which in turn leads to a G _{$\beta\gamma$} -Src-dependent phosphorylation of EGFR, in this case PTX-dependent (Gao et al., 2001).

The ability of bradykinin B₂ receptors to induce tyrosine phosphorylation of EGFR has been described in COS-7 cells, transiently transfected with bradykinin B₂ receptor. In these cells BK employed two independent pathways to activate ERK1/2: a PKC-dependent pathway and EGFR transactivation, although the mechanism of the latter was not elucidated (Adomeit et al., 1999). BK-induced transinactivation of EGFR by stimulation of protein-tyrosine phosphatase was shown in epidermoid carcinoma cells A431. Interestingly, in these cells BK simultaneously activated ERK1/2 independent of EGFR (Graness et al., 2000).

Our results suggest that the mechanism of the B₂ receptor-induced transactivation of EGFR in mIMCD-3 cells is different from those described in the

literature, for it does not involve a complex formation between the B₂ receptor and EGFR and does not require PTX-sensitive G proteins, Ca²⁺, CaM, PLC, or PKC activity (Figure 14). The precise mechanism of BK-induced activation of EGFR in mIMCD-3 cells remains unclear. This novel mechanism may involve Ca²⁺-independent activation of non receptor tyrosine kinase Src or the release of EGFR ligand. Future studies are required to examine these possibilities.

In summary, a cultured murine cell model (mIMCD-3) of the inner medullary collecting duct was used to examine the regulation of ERK1/2 by BK. In this study for the first time, we provide evidence that BK stimulates early mitogenic signals associated with activation of ERK1/2 in mIMCD cells. First, we demonstrated that bradykinin B₂ receptor stimulates Ras-dependent activation of ERK1/2 in mIMCD-3 cells via a novel mechanism of EGFR transactivation. Second, we showed that BK-stimulated proliferation of mIMCD-3 depends on activation of EGFR tyrosine kinase and ERK1/2 (Figure 13). Although the exact nuclear events linking BK-induced ERK1/2 activation with DNA synthesis in mIMCD-3 cells are still to be defined, the findings of the present study contribute to better understanding of the molecular and cellular mechanisms by which BK stimulates proliferation of kidney cells, which could lead to the development of new strategies for treatment of kidney diseases.

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Footnotes

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Legends for figures

Figure 1. *Bradykinin-induced ERK1/2 phosphorylation: time course*

- A. mIMCD-3 cells were stimulated with 10 nM BK for the indicated periods of time. ERK1/2 phosphorylation was measured by immunoblotting with anti-phospho-ERK1/2 antibodies as described in Methods. This is a representative immunoblot (phospho-ERK). The same blot was stripped and re-probed with antibodies for total ERK1/2 that recognize ERK1/2 independently of phosphorylation state to assure an equal loading of a protein samples on a gel (total ERK).
- B. Data points represent intensities of phospho-ERK1/2 bands relative to total-ERK expressed as percent of control (samples without BK treatment). Experiments were performed at least 3 times. Data are shown as mean $\pm$ S.E.M.

Figure 2. *Bradykinin-induced ERK1/2 phosphorylation: concentration response curve*

- A. mIMCD-3 cells were stimulated with the indicated concentrations of BK for 5 minutes. ERK1/2 phosphorylation was detected by immunoblotting with anti-phospho-ERK1/2 antibodies as described in Methods. A representative phospho-ERK1/2 immunoblot is shown. The same blot was stripped and re-probed with antibodies for total ERK1/2 that recognize ERK1/2 independently

of phosphorylation state to assure an equal loading of a protein samples on a gel (total ERK).

B. Data points represent intensities of phospho-ERK1/2 bands relative to total ERK1/2 measured by densitometry and expressed as percent of control (samples without BK treatment). Experiments were performed at least 3 times. Data are shown as mean $\pm$ S.E.M.

Figure 3. *Bradykinin-induced ERK1/2 phosphorylation is mediated by B₂ receptor*

mIMCD-3 cells were pretreated for 30 minutes with the B₁ receptor antagonist des-Arg¹⁰-HOE-140 (1 μ M) or with the B₂ receptor antagonist HOE-140 (1 μ M), then stimulated with 100 nM of BK for 5 minutes. ERK1/2 phosphorylation was detected by immunoblotting with anti-phospho-ERK1/2 antibodies as described in Methods. Bars represent intensities of phospho-ERK1/2 relative to total ERK1/2 expressed as percent of control (cells without BK treatment) phosphorylation. Experiments were performed 3 times in duplicates. Data are presented as mean $\pm$ S.E.M. Inset shows a representative phospho-ERK1/2 blot and the same blot, stripped and re-probed with antibody for total ERK1/2.

Figure 4. *BK receptors do not couple to G_{i/o} proteins in mIMCD-3 cells.*

mIMCD-3 cells were preincubated with vehicle or PTX (200ng/ml overnight), then stimulated with 10 nM BK or 1 μ M LPA for 5 minutes. ERK1/2 phosphorylation was detected by immunoblotting with anti-phospho-ERK1/2 antibodies as

described in Methods. Bars represent intensities of phospho-ERK1/2 relative to total ERK expressed as percent of control (cells without treatment) phosphorylation. Experiments were performed 2 times in duplicates. Data are presented as mean $\pm$ S.E.M. * $P < 0.05$ vs. control.

Figure 5. *Bradykinin-induced ERK1/2 phosphorylation is MEK1/2-dependent.*

mIMCD-3 cells were pretreated for 30 minutes with 10 μ M of PD 098059 and with 10 μ M of U 0126 for 30 minutes prior to stimulation with 10 nM of BK for 5 minutes. ERK1/2 phosphorylation was detected by immunoblotting with anti-phospho-ERK1/2 antibodies as described in Methods. Bars represent intensities of phospho-ERK1/2 relative to total ERK1/2 expressed as percent of control (cells without treatment) phosphorylation. Experiments were performed 3 times in duplicates. Data are presented as mean $\pm$ S.E.M. Inset shows a representative phospho-ERK1/2 blot and the same blot, stripped and re-probed with antibody for total ERK1/2.

Figure 6. *Bradykinin-induced ERK1/2 phosphorylation is PKC- and PLC-independent.*

A. mIMCD-3 cells were preincubated with 1 μ M of GF109203X for 30 minutes or with 160 nM of PMA for 20 hours, followed by either BK (10 nM) or PMA (1 μ M) stimulation for 5 minutes. ERK1/2 phosphorylation was detected by immunoblotting with anti-phospho-ERK1/2 antibodies as described in Methods. Bars represent intensities of phospho-ERK1/2 relative to total

ERK1/2 expressed as percent of control (cells without treatment) phosphorylation. Experiments were performed 3 times in duplicates. Data are presented as mean $\pm$ S.E.M. ** $P < 0.01$ vs. control.

B. mIMCD-3 cells were preincubated with vehicle or with 3 different PLC inhibitors U-73122 (10 μ M), D609 (50 μ M), and ET-18-OCH₃ (50 μ M) for 30 minutes and then stimulated with 10 nM BK for 5 minutes. Experiments were performed at least 4 times in duplicates. Data are presented as mean $\pm$ S.E.M.

Figure 7. *Bradykinin-induced ERK1/2 phosphorylation is Ca²⁺- and CaM-independent.*

mIMCD-3 cells were pretreated for 30 minutes with vehicle, 10 μ M of BAPTA, or with 50 μ M of fluphenazine and then stimulated with 10 nM BK for 5 minutes. ERK1/2 phosphorylation was detected by immunoblotting with anti-phospho-ERK1/2 antibodies as described in Methods. Bars represent intensities of phospho-ERK1/2 relative to total ERK1/2 expressed as percent of control (cells without BK treatment) phosphorylation. Experiments were performed at least 3 times in duplicates. Data are presented as mean $\pm$ S.E.M.

Figure 8. *Role of PI3K and tyrosine kinases in BK-induced ERK1/2 phosphorylation.*

mIMCD-3 cells were pretreated for 30 minutes with vehicle or with two different specific inhibitors of PI3K (50 nM wortmannin and 50 μ M LY294002) or with

tyrosine kinase inhibitors (50 μ M of genistein or 50 μ M of its negative control daidzein, 50 μ M of AG-490, or 100 nM of AG1478) before stimulation with 10 nM BK for 5 minutes. ERK1/2 phosphorylation was detected by immunoblotting with anti-phospho-ERK1/2 antibodies as described in Methods. Bars represent intensities of phospho-ERK1/2 relative to total ERK1/2 expressed as percent of control (cells without BK treatment) phosphorylation. Experiments were performed at least 3 times in duplicates. Data are presented as mean $\pm$ S.E.M. ** $P < 0.01$ vs. control.

Figure 9. *Bradykinin induces phosphorylation of EGF receptor.*

Inset: *The time course for EGFR phosphorylation.* Cells were stimulated with 10 nM BK for the indicated periods of time. EGFR phosphorylation was measured as described in Methods. Data points represent intensities of phosphorylated EGFR bands measured by densitometry and expressed as fold increase over control (non stimulated cells). A representative phospho-EGFR immunoblot is shown at the top part of inset. Same blots were stripped and re-probed with antibodies for total EGFR that recognize EGFR independently of phosphorylation state to assure an equal loading of a protein samples on a gel (data not shown). Experiments were performed 2 times in duplicates. Data are presented as mean $\pm$ S.E.M.

BK-induced EGFR phosphorylation depends on EGFR tyrosine kinase activity. mMCD-3 cells were preincubated for 30 minutes with vehicle or with 100 nM of

AG1478, and stimulated for 5 minutes with 10 nM of BK or with 10 ng/ml EGF.

EGFR phosphorylation was measured as described in Methods.

A. This is a representative phospho-EGFR immunoblot.

B. Bars represent intensities of phospho-EGFR bands measured by densitometry and expressed as fold increase over control (without BK treatment) phosphorylation. Experiments were performed at least 3 times in duplicates.

Data are presented as mean $\pm$ S.E.M. ** $P < 0.01$ vs. control.

Figure 10. *Immunoprecipitation/Western blotting studies do not support complex formation of B₂ receptor with EGFR.* Cells were stimulated for 5 minutes with 10 nM BK or 1ng/ml EGF. Immunoprecipitation of cell lysates with anti-EGFR antibody followed by immunoblotting with anti-B₂ receptor and/or with anti-phospho-EGFR antibody were performed as described in Methods.

A. Representative phospho-EGFR immunoblot. Arrow shows a band corresponding to phosphorylated EGFR. The same blot was stripped and re-probed with anti-EGFR antibody to assure an equal loading of a protein samples on a gel (data not shown).

B. Representative B₂ receptor immunoblot. Arrow points to a band corresponding to B₂ receptor in a positive control lanes. As a positive control we used 20 μ g of total protein of non stimulated mIMCD-3 cell lysates per lane. The same blot was stripped and re-probed with anti-EGFR antibody to assure that EGFR was indeed immunoprecipitated (data not shown). Experiments were performed 3 times.

IP, immunoprecipitation; *IB*, immunoblot.

Figure 11. *BK stimulates complex formation of Grb2 with EGFR and association of Grb2 with Shc in mIMCD-3 cells.*

A. *BK stimulates complex formation between EGFR and Grb2.* Cells were stimulated for 5 minutes with 10 nM BK or 1ng/ml EGF. Immunoprecipitations of cell lysates with anti-EGFR antibody followed by immunoblotting with anti-Grb2 antibody were performed as described in Methods. Bars represent intensities of Grb2 bands measured by densitometry and expressed as percent of control (non treated samples). Experiments were performed 3 times. Data are presented as mean $\pm$ S.E.M. * $P < 0.05$ vs. vehicle.

B. *BK induces association of Grb2 with Shc.* Cells were stimulated for 5 minutes with 10 nM BK or 1 ng/ml EGF. Immunoprecipitations of cell lysates with anti-Shc antibody followed by immunoblotting with anti-Grb2 antibody were performed as described in Methods. Bars represent intensities of Grb2 bands measured by densitometry and expressed as percent of control (non-treated samples). Experiments were performed 3 times. Data are presented as mean $\pm$ S.E.M. * $P < 0.05$ vs. vehicle.

IP, immunoprecipitation; *IB*, immunoblot.

Figure 12. *BK activates Ras in mIMCD-3 cells.*

A. *Time course for BK-induced Ras activation.* mIMCD-3 cells were stimulated with 10 nM BK for indicated periods of time. Ras activity was measured using

Ras activation kit as described in Methods. Data points represent intensities of Ras immunoreactive bands measured by densitometry and expressed as percent of control (samples without BK treatment). Experiments were performed 2 times in duplicates. Data are shown as mean $\pm$ S.E.M.

B. mIMCD-3 cells were pretreated with vehicle or 100 nM AG1478 for 30 minutes and then stimulated with 10 nM BK or 1 ng/ml EGF for 5 minutes. Ras activity was measured using Ras activation kit as described in Methods. Bars represent intensities of Ras immunoreactive bands measured by densitometry and expressed as percent of control (non treated samples). Experiments were performed at least 3 times in duplicates. Data are shown as mean $\pm$ S.E.M. * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

Figure 13. *BK-induced DNA synthesis in mIMCD-3 cells depends on EGFR and ERK1/2 activity.* Serum-deprived mIMCD-3 cells grown in 24-well plates were preincubated for 1 hour with 50 μ M PD98059, 100 nM AG1478, or vehicle before addition of 100 nM BK or 10 ng/ml EGF for 24 hours. [3 H]thymidine was included in final 6 hours of incubation, and [3 H]thymidine incorporation into DNA was measured as described in Methods. Experiments were performed at least 4 times in triplicates. Data are shown as mean $\pm$ S.E.M. * $P < 0.05$ vs. vehicle treated cells; ** $P < 0.01$ vs. vehicle treated cells; ζ $P < 0.05$ vs. control; $\zeta\zeta$ $P < 0.01$ vs. control.

Figure 14. *Proposed model for BK-induced ERK1/2 activation.* BK stimulates EGFR transactivation through the B₂ receptor-G_{q/11} pathway and subsequent Ras-dependent activation of ERK1/2. The mechanism of BK-induced transactivation of EGFR is different from published mechanisms of G_{q/11}-coupled receptors-induced EGFR transactivation, in that it does not require PLC, PKC, Ca²⁺/calmodulin as well as direct association of B₂ receptor with EGFR. We speculate that this novel mechanism may involve Ca⁺²-independent activation of non receptor tyrosine kinase Src or the release of EGFR ligand.

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